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13. ABSTRACT *(Maximum 200 Words)*
It is known that the actions of estrogen in mammary development are mediated primarily by the estrogen receptor alpha (ERα). It is not known whether ERα in epithelium (the tissue in which breast cancer develops) contribute to mammary cell proliferation and ductal development, or whether ERα target genes with classical estrogen response elements (EREs) or with alternative response elements, especially AP-1 and CRE elements, mediate proliferation. We have been successful in developing transgenic mice with expression of wild type and AP-1/CRE superactive human ERα (K206A) in mammary gland and reproductive track (vaginal-cervical) epithelium. We use d the keratin 14 gene promoter to drive expression in mammary basal epithelial cells and cervical- vaginal epithelium, and the MMTV promoter to drive expression throughout the mammary epithelium. In the K14 transgenics, expression in the genital tract was efficient and caused hyperproliferation, cyclin D1 over-expression and organ enlargement. Expression of human ERs in the mammary basal cells was less strong, but excessive proliferation and lobular development occurred after estrogen treatment in these animals. We have applied for funding from NIH to finish the study of the mammary gland in these animals and in the MMTV transgenics, which appear to have good expression of the transgene in mammary glands. We tentatively conclude that human ERα can function to mediate proliferation in the epithelial cells of the reproductive track and mammary gland, and that the target genes with AP-1/CRE elements are important in this process. If further studies confirm these observations it will suggest that the ERα pathway to AP-1/CRE target genes is a key target for interventions to prevent breast cancer.

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Introduction:

Our goal in this research project is to understand the exact pathways by which estrogen controls mammary ductal proliferation and development. It is known that the actions of estrogen in mammary proliferation are mediated primarily by estrogen receptor alpha (hERα), and not by estrogen receptor beta (ERβ). It is not known whether ERα in mammary epithelium (which is the tissue in which breast cancer develops) contributes to the proliferation, or whether ERα in mammary stroma mediate all the effects. In addition, ERα works through two pathways, regulating target genes with classical estrogen response elements (EREs) and target genes with alternative response elements, such as AP-1 and CRE sites. We wish to know whether epithelial ERα contributes to estrogen mediated proliferation and whether the ERE or the AP-1/CRE pathway is most important. Our specific plan is to probe this question by constructing transgenic mice in which wild type human ERα and mutants of the receptor that are selectively super-active at the alternative AP-1/CRE pathway are expressed in mammary gland other estrogen responsive epithelia. We will then compare proliferation in the transgenics and non-transgenic controls. We are especially keen to determine whether overexpression of human ERs in epithelium increases proliferation, and whether the wild type human ERα and AP-1 superactive ERα behave the same or differently.

Body-Research Accomplishments, and relationship to tasks:

Research Accomplishments.

There are two parts to the description of research accomplishments: i) We describe how we developed K14 human ERα and K14:K206A mice and discovered that the K206A mice had hyper-proliferation of the reproductive epithelium. A description of this part is under revision for Genes & Development and we include the submitted manuscript for this report. ii) We describe how we have developed transgenic mice with the MMTV promoter driving expression of human ERα and K206A. We also describe how the mammary gland of the K14K206A mice appears to have abnormal lobular-alveolar development after treatment with estrogen.

The mammary glands of the MMTV mice are just now being characterized for mammary epithelial proliferation and development, and we anticipate completing the characterization of the mammary glands of these mice with NIH funding, for which we have applied.

Accomplishments part i.)Hyperproliferation in reproductive track of female transgenicmice with the K14 promoter driving expression the AP-1/CRE selectively superactive mutant, K206A.

An estrogen receptor that is superactive at alternative response elements causes hyper-proliferation

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Estrogen stimulates proliferation via estrogen receptor alpha (ERα), which activates expression of target genes with classical estrogen response elements (EREs), or with alternative response elements including AP-1 and variant CRE sites. We show that a point mutant in human ERα, K206A, is superactive at AP-1/ CRE sites and underactive at EREs. Targeted expression of ERαK206A, but not of ERα, in female mouse genital tract causes enlargement of the vagina and cervix and hyper-proliferation. Expression of cyclin D1, an alternate response gene is elevated in ERαK206A transgenics, whereas expression of lactoferrin, an ERE gene, is not. These observations implicate the ERα pathway to alternative response elements in estrogen-induced proliferation.

Introduction

Estrogen stimulates proliferation of epithelial cells in the reproductive tract and mammary gland of females, and in the prostate of males, via estrogen receptor alpha (ERα), which functions as transcription factor to regulate expression of target genes (Parker 1998). Female mice in which ERα has been knocked-out (αERKOs) lack estrogen-induced proliferation of the uterus, cervix and vagina (Lubahn et al. 1993; Korach 1994). Male αERKO mice are completely resistant to estrogen-induced prostate metaplasia and cancer (Risbridger et al. 2001). In contrast, both male and female mice lacking the second estrogen receptor, ERβ, have normal or even hyper-normal responses to estrogen (Weihua et al. 2001).

ERα works by modulating the transcription of target genes, whose products are thought to be responsible for estrogen-dependent phenotypes such as the proliferation and development of estrogen-regulated target tissues. However, the identity of many of the specific estrogen-regulated genes that regulate proliferation, and the mechanism of their regulation, is not yet clear. In general, ERα activates expression of two types of target genes. One type has classical estrogen response elements (EREs) in the promoter region which allow the receptor to bind via its centrally located DNA binding domain, and then to recruit coactivator complexes that remodel chromatin and switch on the transcriptional machinery (Parker 1998). A second type of ERα target genes has alternate response elements, which bind heterologous transcription factors but not ERα. This category includes AP-1 sites that bind a Jun/Fos complex (Gaub et al. 1990; Philips et al. 1993;

Kushner et al. 2000), or variant cyclic-AMP response elements(CREs) that bind a Jun/ATF-2 complex (Sabbah et al. 1999), or Sp1 sites that bind Sp1 (Saville et al. 2000). The DNA of alternative response elements does not bind ERα, which is believed to participate via protein-protein interactions with the heterologous transcription factors that are bound to these elements or their coactivators (Webb et al. 1995; Webb et al. 1999; Kushner et al. 2000).

Presently, the significance of ERα action at alternate response elements is not understood. Some correlative evidence, however, has pointed towards the importance of the ERα-AP-1 pathway in estrogen effects on proliferation. When the partial agonist tamoxifen mimics estrogen and stimulates cell proliferation, it also stimulates AP-1 but not necessarily ERE transactivation. (discussed in (Webb et al. 1995; Paech et al. 1997; Philips et al. 1998; Webb et al. 1999; Kushner et al. 2000)). Moreover, ERα can either stimulate or repress the growth response of different breast cell lines in cell culture, and the nature of this response correlates with the effects of ERα on AP-1 activity in the same cell types (Philips et al. 1998). Other correlative evidence comes from the study of specific target genes believed to mediate the proliferative effects of estrogen in the mammary gland. One of the key genes mediating such effects is cyclin D1, which is induced by estrogen, but has no ERE. Induction of cyclin D1 is transcriptional and appears to be mediated mainly by a variant CRE element that binds the AP-1 related factors Jun/ATF-2(Sabbah et al. 1999).

Here, we test the hypothesis that ERα action at alternative response elements is important for estrogen-dependent proliferation in vivo. We describe a mutant of human ERα that is selectively superactive at target genes with alternative response elements, but not at target genes with classical EREs. We explore the effect of the superactive receptor on proliferation by targeting expression to the epithelium of the genital tract of female mice.

Results

ERα K206A is superactive at target genes with alternative response elements.

To probe the role of the ERα/AP-1 pathway in vivo, we looked for an ERα mutation that would affect action at AP-1 sites and other alternate response elements without affecting action at classical EREs. It has previously been shown that a mutation in a conserved lysine residue at the base of the first zinc finger of the DNA binding domain converts the glucocorticoid and thyroid hormone receptors from inhibitors to activators at AP-1 without affecting their action at classical hormone response elements (Starr et al. 1996). We therefore investigated the phenotype of a similar point mutation in hERα (K206A, Fig. 1A).

We first examined the activity of the mutant ERα on reporter genes with different response elements in HeLa cells. In accordance with our previous results, hERα stimulated transcriptional activity on an AP-1 reporter (human collagenase promoter) by two to five fold

(Fig.1B). However, in parallel hERαK206A was 5 to 200 fold more active than wild type hERα. This phenotype was also observed on a range of AP-1 responsive reporter genes (not shown) and in different cell types (Fig. 1C). As noted above, the cyclin D1promoter does not have an ERE and responds to hERα mainly through a variant CRE that binds AP-1 related proteins and is located near the promoter with minor contribution of a more distant consensus AP-1 site (Altucci et al. 1996; Sabbah et al. 1999). Again, hERαK206A was superactive on the cyclin D1 promoter compared to wild type hERα (Fig. 1B). As expected, these estrogen effects required the integrity of both the CRE and AP-1 sites (in preparation). In contrast, the hERαK206A mutation seemed to reduce the efficiency of ERα action at a promoter with Sp1 sites (Saville et al. 2000; Safe 2001). Thus, ERaK206A super-activates a subset of estrogen-regulated promoters with alternate response elements.

The super-activity of K206A is selective and does not extend to target genes with classical EREs. We then examined the effect of the hERαK206A mutation at reporter genes with classical EREs. ERαK206A had modestly reduced activity at the lactoferrin and pS2 gene promoters which are regulated by near-consensus EREs (Nunez et al. 1989; Liu and Teng 1992) (Fig. 2). ERα K206A also failed to super-activate at a promoter in which a consensus classical ERE replaced the AP-1 site in collagenase, nor at several target genes with synthetic consensus EREs (data not shown).

Thus, hERK206A selectively super-activates target genes that, like cyclin D1, have AP-1 sites or variant CRE sites and but not target genes with EREs.

Expression of ERαK206A causes enlargement in mouse genital tract.

To determine the effect of super-activation at AP-1 and CRE sites (hereafter AP-1/CRE sites) within an intact organism, we targeted expression of hERαK206A or wild type hERα to the squamous epithelium of the female genital tract, an estrogen responsive tissue. We used the human keratin-14 gene promoter, which allows expression in basal epithelial cells of the vagina and cervix, in basal epithelial cells of the glandular uterus, but not in the luminal epithelial cells of the uterus ((Arbeit et al. 1996), and data not shown). Two independent transgenic mouse lines were established for each type of transgenic (K14-hERα, and K14-hERαK206A) and observed for up to 24 months of age. Thirty of 36 female mice from the two lines of K14-hERαK206A mice spontaneously developed perineal swelling beginning at 6 months of age, which on examination of internal organs was due to marked vaginal and cervical enlargement (Fig. 3, top row). The vagina in K14-hERαK206A transgenics was folded in pleats, and the vaginal and cervical squamous epithelium was thickened, papillomatous, and hyperplastic (Fig. 3 bottom row, quantitative data not shown). No abnormalities occurred in the uterine luminal epithelium, which does not express the transgene, but the uterine glands, which do express the transgene, are enlarged (not shown). K14-hERα transgenics, by contrast, had normal reproductive tracts (Fig. 3,

bottom row center). Expression of human estrogen receptors in the squamous epithelium of the K14-hERαK206A transgenics was not greater than in the K14-hERα transgenics (Fig. 4, top row). Thus the abnormal vaginal-cervical phenotype of K14-hERαK206A transgenics is a feature of the mutation.

K14-hERαK206A transgenics exhibit hyper-proliferation and over-expression of cyclin D1,with normal expression of lactoferrin.

To determine whether the hERαK206A genital enlargement was associated with increased cellular proliferation, the pattern, distribution, and expression level of proliferating cell nuclear antigen (PCNA) and cyclin D1 were analyzed (Fig.4, second and third rows). The frequency and expression level of PCNA was increased in both basal and suprabasal squamous epithelial cells in the K14-hERαK206A transgenic mice, but not in wild type hERα transgenics. Cyclin D1 expression was also elevated in the K14-hERαK206A transgenics, but not in the wild type ERa transgenics. Moreover, the pattern of cyclin D1 overexpression was identical to the distribution of PCNA suggesting that these two markers of proliferation are up-regulated in the same cells. Therefore, increased cyclin D1 expression correlates with the increased cell cycle activity and hyperplasia in the cervical and vaginal squamous epithelium of K14-hERαK206A transgenic mice. This is consistent with other studies that have shown a role for cyclin D1 up-regulation in

estrogen stimulated proliferation in mammary cells in culture and in mice (Sicinski et al. 1995; Prall et al. 1998). Expression of lactoferrin, a classical ERE regulated gene, is no greater in K206A transgenics than in wild type transgenics, consistent with the selective super-activity of the mutant receptor. In summary, genital tract enlargement in female hERaK206A transgenics is associated with hyper-proliferation and overexpression of cyclin D1, an alternative target gene, without overexpression of a classical target gene.

Discussion

While it is clear that estrogens regulate proliferation through ERα, it is not clear which ERα target genes mediate these proliferative effects, nor how ERα regulates these genes. These studies show that mutation of the conserved lysine, K206, at the base of the first zinc finger in the DNA binding domain of hERα profoundly changes its ability to activate different types of target genes. The mutant, hERαK206A, activates target genes with classical EREs with a similar efficiency to wild type hERα, but selectively super-activates target genes with AP-1 or CRE sites. This pattern occurs with a variety of promoter contexts containing either EREs or AP-1/CRE sites and in several different types of transfected cells in culture.

The pattern of activation is also preserved in vivo, as the hERαK206A mutant leads to the overexpression of cyclin D1, an AP-1/CRE regulated gene, in transgenic genital tract but not

overexpression of lactoferrin, an ERE regulated gene. Targeted expression of ERαK206A, but not wild type ERα, in female genital tract leads to enlargement and hyper-proliferation as noted by dramatic increases in PCNA staining and cyclin D1 overexpression. Interestingly, hyper-proliferation is restricted to tissues expressing the transgene. Thus, the uterine luminal epithelium, which is highly sensitive to estrogen provoked proliferation, but which does not allow expression of the transgene, is normal in these transgenics. The uterine glands, which do express the transgene, are enlarged. Together these observations suggest that the selective action of hERαK206A at AP-1/CRE sites is responsible for the hyper-proliferation of the reproductive tract and, in turn, suggests that the ERα pathway to AP-1/CRE sites is important for proliferation in vivo.

While our evidence is consistent with the notion that ERαK206A causes hyperproliferation by super-activating genes with alternate response elements, the identity of these estrogen-regulated genes is not clear. We have seen that one candidate gene that contains alternate response elements, cyclin D1, is overexpressed. Because the pattern of staining for PCNA and cyclin D1 appear to be the same, hyper-proliferation may stem, in part, from overexpression of cyclin D1. Previous studies with MMTV:cyclin D1 and K5:cyclin D1 transgenic mice revealed hyper-proliferation in mammary gland and reproductive epithelia respectively, suggesting that cyclin D1 overexpression was sufficient for hyper-proliferation (Wang et al. 1994; Robles et al. 1996).

However it is also possible that the hERαK206A transgene additionally causes overexpression of other D-type cyclins or other pro-proliferative genes with a similar function.

Our studies do not address the reasons that ERαK206A super-activates genes with AP-1 sites. The conserved lysine, K206, appears to be a unique site for mutations of hERα that confer super-activity at AP-1 sites while preserving activity at EREs (data not shown). Likewise, the homologous residue in the glucocorticoid receptor, K461, is the only residue among 30,000 tested mutants of the DNA binding domain that can will confer a similar profound change of target gene preference at AP-1 sites (Starr et al. 1996). We show elsewhere that the mutant super-activates AP-1/CRE target genes through its AF-1 and AF-2 functions, and suggest that the mutation may relieve inhibition mediated by the DNA binding domain when the receptor is not bound to DNA (R Uht et al. in preparation).

Finally, we speculate that ERα action at alternate response elements will prove to be important for estrogen-dependent proliferation in other settings. If further studies with K206A transgenics that allow expression in the uterus, mammary gland, ovary, and prostate confirm hyper-proliferation, it will suggest a potential role for over-stimulation of the ER-AP-1/CRE pathway in the pathogenesis of estrogen induced proliferative disorders. Since the ER-AP-1/CRE pathway has distinguishing functional requirements (Webb et al. 1999), such studies would suggest it as a potential target for drugs that disrupt the pathway and that might be useful for cancer prevention.

Materials and Methods

Plasmids

Reporters genes driven by the collagenase promoter and by the cyclin D1 promoter were previously described (Albanese et al. 1995; Webb et al. 1995). HSV-TK:LUC contains Herpes Simplex Virus TK promoter sequences –109/+45 which contains two SP-1 sites. and was prepared by removing the consensus ERE from ERE II TK:LUC (Webb et al. 1995). hER and hERG400V expression vectors were previously described (Webb et al. 1995). The K206 mutation was introduced by site-directed mutagenesis (Quickchange kit, Stratagene). To construct transgene expression vectors hERα and hERα.K206A were isolated by EcoR1 digest, converted to blunt ends with Klenow and ligated into a K14 transgene vector (Munz et al. 1999) digested with SmaI.

Transfections

Transfection were performed by electroporation using 2μg reporter, 1μg actin-βgalactosidase expression vector as an internal control and 5μg ER expression vectors or empty vector (Webb et al. 1995). Luciferase and βgal activities were determined by standard methods 36-40hrs after plating. To calculate relative luciferase activity, luciferase activities were divided by βgal activity and the value obtained in the absence of ER expression vector and estradiol was set at one.

Histopathology and immunohistochemistry

As described previously (Arbeit et al. 1996) mice were sacrificed by perfusion of the ascending aorta with 3.75% paraformaldehyde under Avertin anesthesia. Reproductive tracts were dissected and post-fixed overnight at 4°C. After removal of the posterior vaginal wall, tissues were rinsed in phosphate buffered saline (PBS), dehydrated through graded alcohols and xylene, embedded in paraffin, and 5um sections were stained with hematoxylin/eosin (Sigma). PCNA immunohistochemistry was carried out as described previously (Arbeit et al. 1996). Briefly, 5um

tissue sections were deparaffinized, rehydrated and subjected to antigen retrieval in 10mM citrate buffer, pH 6.0 by microwave for two 5-minute high-power pulses. Sections were blocked in 3% bovine albumin (Sigma) in PBS, and subsequently-incubated with a 1:200 dilution of mouse anti-PCNA monoclonal antibody (Biogenex). Signal development was performed by using a biotinylated goat anti-mouse IgM secondary antibody (diluted 1:200; Vector), the Vector Elite immunoperoxidase reagent (Vector), and NovaRed solution (Vector) as a substrate. Sections were counterstained with Gill's #1 hematoxylin (Sigma). Human ERα IHC was carried out similarly, using a 1:200 dilution (in 0.2% bovine albumin) of anti-human ERα monoclonal antibody D75 (Greene et al. 1980) and biotinylated anti-rat IgG secondary antibody (1:200 in 0.2% bovine albumin; Vector). Immunohistochemistry for cyclin D1 (1:500; Upstate Biotechnology) and Lactoferrin (1:2000; a generous gift from C.T. Teng, NIEHS, North Carolina) was performed as described for PCNA, but additionally included blocking of endogenous peroxidase by a 20-minute incubation in 3% hydrogen peroxide in methanol following tissue dehydration. A 1:200 dilution of a biotinylated anti-rabbit IgM (Vector) was followed by 3,3'-diaminobenzidine (Sigma) as the chromogen.

Acknowledgements

We thank Geoffrey Greene for the D75 antibody, Richard Pestell and Sabina Werner for plasmids, and David Elson, Kristin Hilty and Carol Anderson for technical assistance. PJK is a consultant, and has significant financial interests in KaroBio AB, a Swedish company developing drugs that target nuclear receptors.

Figure legends

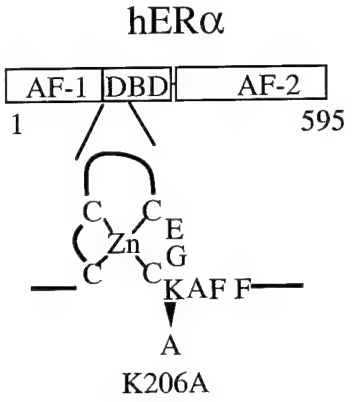
Figure1 A point mutation in the ERα DNA binding domain (DBD) results in super-activation at promoters regulated by estrogen through AP-1/CRE sites. A, Location of the hERαK206A mutation in the first zinc finger of hERα B, Ability of the hERαK206A mutant to enhance expression of a reporter genes regulated by estrogen through an AP-1 site (human collagenase promoter), CRE (cyclin D1), or Sp1 site (HSV-TK). C, hERαK206A super-activity on an AP-1 reporter in a variety of cell lines in culture: HeLa, cervical cancer; Ishikawa, endometrial cancer; MDA-MB & MCF-7, breast cancer; DU145, prostate cancer; GT1, hypothalamic; GHT1-5, pituitary lactotroph; CEF, chick embryo fibroblasts; COS, SV40 transformed kidney fibroblast.

Figure 2 The hERαK206A mutant is underactive at target genes with classical EREs. Ability of the mutant and wild type hERs to activate expression of reporter genes with the lactoferrin and pS2 promoters, which contain near consensus EREs, and at a promoter with a consensus ERE replacing the AP-1 site of collagenase (ERE-Coll),

Figure 3 Reproductive tract abnormalities in female K14-hERαK206A transgenic mice. A, Vaginal enlargement (but note normal uterus) develops in K14-hERαK206A transgenic (right) but not in age-matched (9 months) nontransgenic (left) or wild type K14-hERα transgenic (center) mice. B, K14-hERαK206A mice develop cervical and vaginal enlargement with squamous epithelial thickening not seen in nontransgenic (left) or K14-hERα transgenic (center) mice.

Figure 4 Hyper-proliferation in K14-hERαK206A transgenic genital epithelium. First row shows that human ERα (huERα), assayed with a specific monoclonal antibody, is not more strongly expressed in vaginal squamous epithelium of K14-hERαK206A transgenic mice (right) than in K14-hERα transgenic (center) or nontransgenic (FVB/n, left) control mice (200x). Second row shows that ERαK206A transgenic mice have more proliferating cells than ERα transgenic or nontransgenic mice as revealed by staining for proliferating cell nuclear antigen (PCNA) in both basal and suprabasal cell layers (400x). Third row shows that cyclin D1 immunoreactivity is increased and detected in multiple basal and suprabasal vaginal epithelial cell layers in ERαK206A transgenic mice, compared to low-level basal cell expression in ERα transgenic and nontransgenic mice (400X). Fourth row show that lactoferrin expression is not elevated in the K14-hERαK206A transgenics.

A



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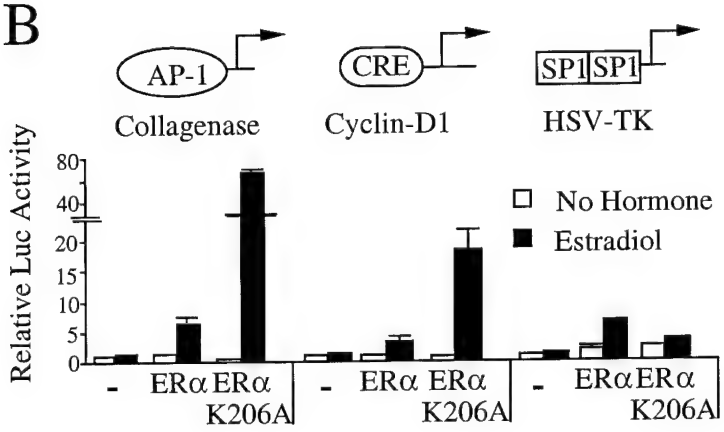
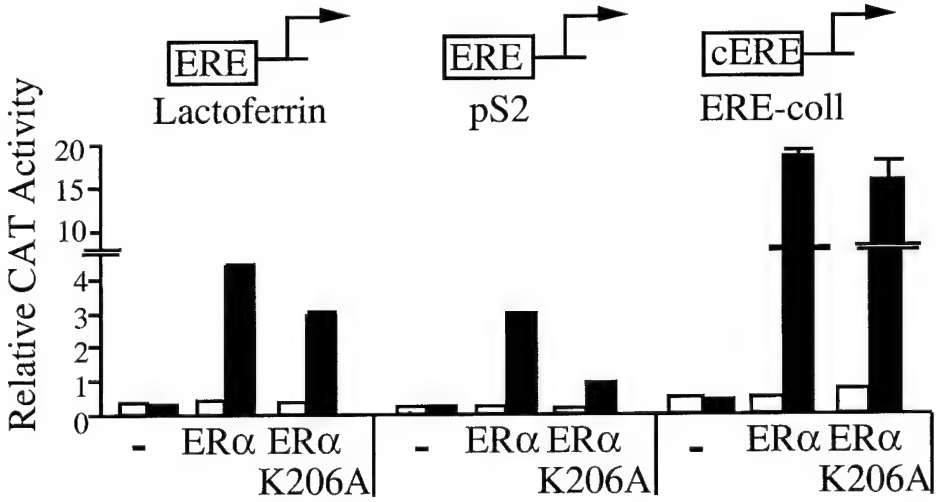


C

	Fold Induction	
Cell Line	hERα	K206A
HeLa	4.3	46.7
Ishikawa	2.1	31.1
MCF-7	2.2	214.3
MDA-MB453	2.2	224.0
MDA-MB231	2.3	81.5
GHFT1-5	1.8	101.1
CEF	1.9	5.2
COS	1.7	7.7

Fig.1


ERE
Lactoferrin
ERE
pS2
cERE
ERE-coll
Relative CAT Activity
20
15
10
4
3
2
1
0
-
ERα
ERα K206A
-
ERα
ERα K206A
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ERα
ERα K206A

Fig. 2

Fig 3

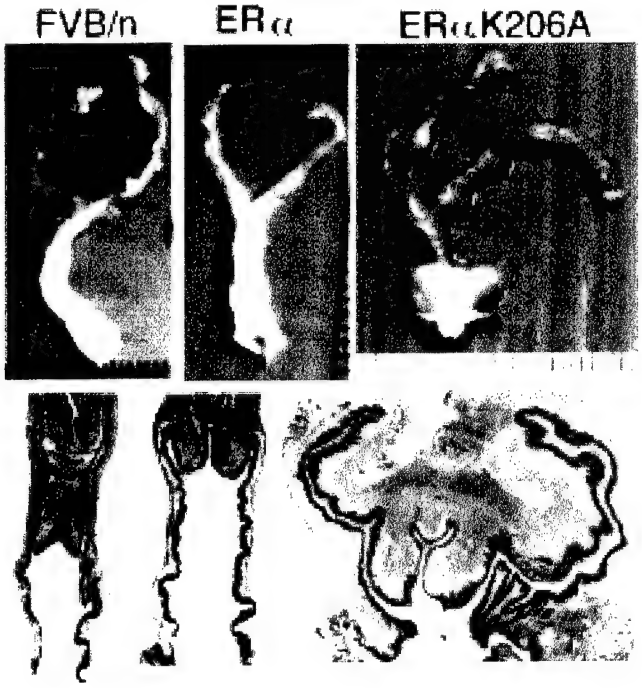
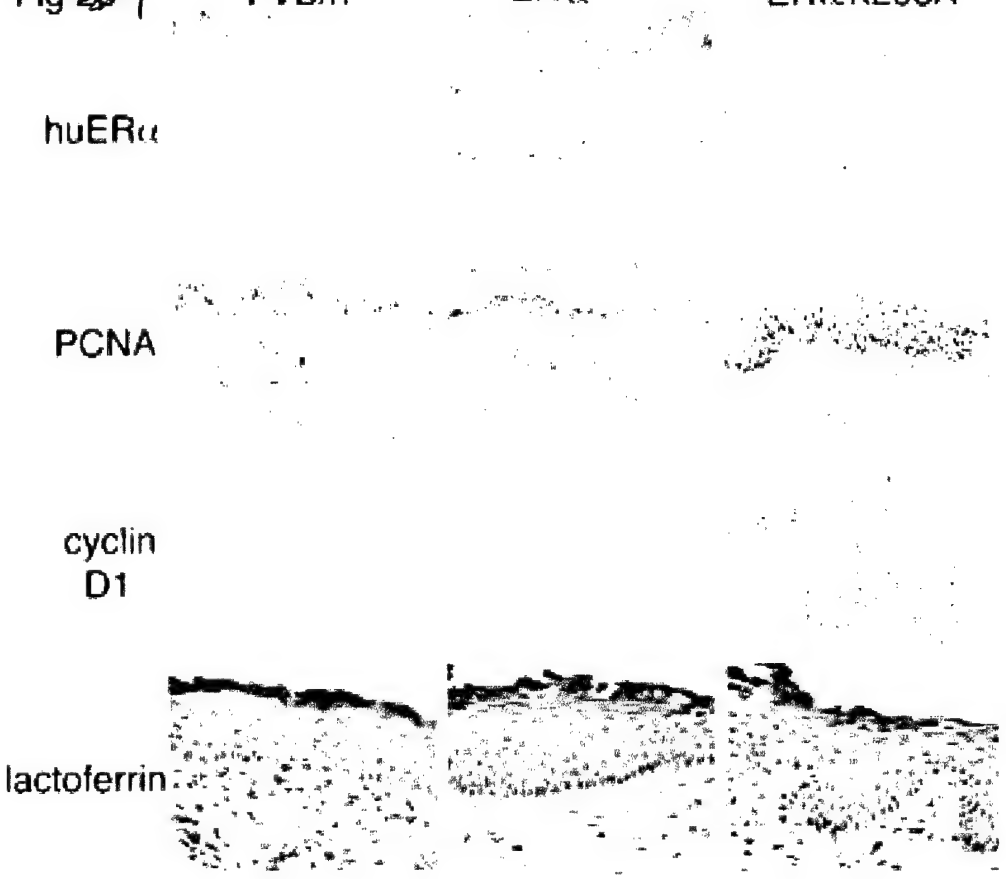

FVB/n
ERα
ERαK206A

Fig 4


FVB/n
ERα
ERαK206A
huERα
PCNA
cyclin
D1
lactoferrin

Accomplishments part ii.) Generation of transgenic mice with MMTV:hER and MMTV:hERK206A, and proliferative abnormalities in the mammary glands of the K14:K206A mice

We produced mice bearing transgenes that target human estrogen receptors (hER) to the mammary epithelial cells. The long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV) was used to drive the tissue specific expression and we have bred numerous litters from the founder mice. Of the originally identified founders, we have focused on two lines of mice expressing the wild-type hER (3H and 7H) and two lines of mice expressing the superactive hER (2K and 3K).

To determine expression levels of the transgene in each of the lines, we have isolated RNA from the mammary glands and carried out RT-PCR using random hexamers to prime the RT reaction and human-specific PCR primers to identify the cDNA from the transgene. While preliminary studies have been carried out (see figure 1 below), we are currently adapting our RT protocol to be used with a Quantitative PCR machine (TaqMan).

To determine if the mammary gland phenotype is normal, we have killed adult, females and dissected the mammary glands and processed the whole mounts for staining. We have only begun to analyze the MMTV-hER mice and the

mammary phenotype at various timepoints across development, pregnancy, lactation and involution. Another set of transgenic mice with the hER and hER-K206A directed to the basal epithelial cells are those bearing the cytokeratin 14 promoter (K14-hER and K14-hER**K206A**). Ongoing is a thorough study of mammary phenotype in virgin females that were either ovariectomized (ovx) and given a placebo pellet or ovx and given an estrogen pellet (0.05 mg). Below are examples of mammary gland phenotype from the noted treatment groups and genotypes.

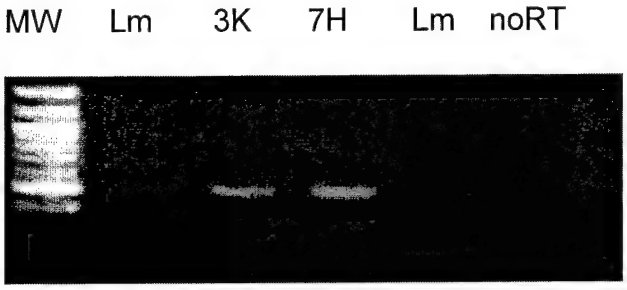


Figure 1: RT PCR analysis of transgene expression in the 3K and 7H lines. Adjacent "Lm" are littermate controls. No RT indicates PCR from an RT reaction that was carried out without RT enzyme.

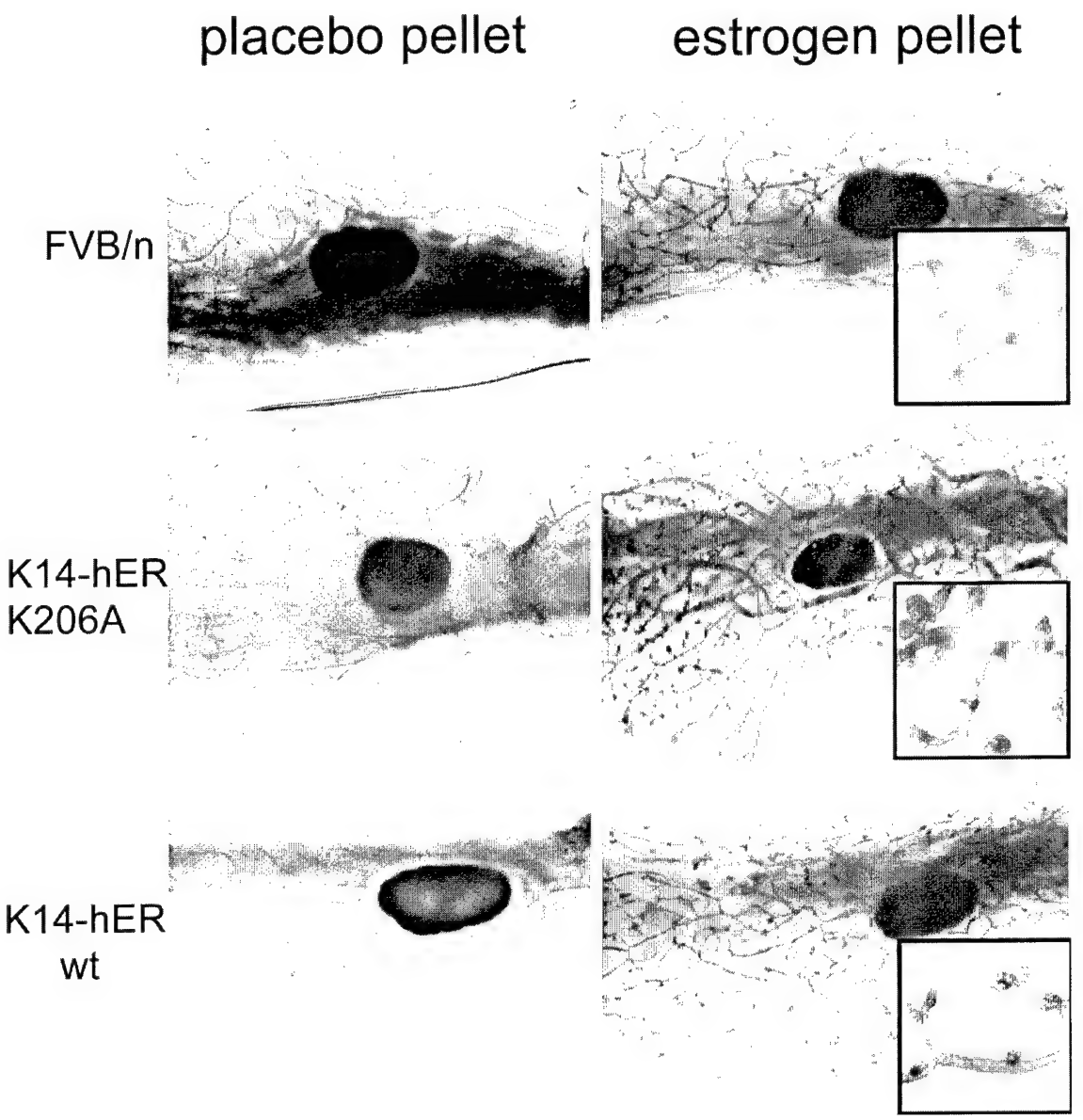


Figure 2: Photographs of mammary glands from ovariectomized mice of either non-transgenic (FVB/n), K14-hER K206A or K14-hER wt. Mice were ovariectomized at 8 weeks and implanted with either placebo or estrogen pellets

(0.05 mg) 4 weeks prior to sacrifice. Insets show a magnified view of relevant features.

Relationship to tasks.

We had initially planned a two phase project. The first phase was to create and characterize transgenic mice with various promoters driving wild type and mutant human estrogen receptors. The second phase was to cross these mice with ERa knock-out mice. We feel that we have accomplished most of the overall goal of the first phase. In particular, we made four lines of transgenic mice and have discovered some profound phenotypes.

Unfortunately, we have not been able to make substantial progress of the second phase. We started to establish an ERa knock-out colony last year, but had to abandon the effort as too expensive with the current level of support. This is the first project using transgenic mice that we have undertaken and we greatly underestimated the expense of working with transgenic mice at UCSF. In addition we were severely limited for space in the mouse house and this forced us to keep to the central core of the project.

Despite the set back with the second task we strongly believe that we had made some invaluable lines of mice for the study of estrogen action in breast cancer development, and we have actually been able to obtain a preliminary answer to the questions we set out to answer. Yes, epithelial estrogen receptors do play a role in estrogen provoked proliferation in the reproductive track and mammary gland. And, yes, the pathway leading to alternative estrogen response elements appears to be important for proliferative effects.

Key Research Accomplishments: Cellular Targets to Estrogen Action,.

- Created transgenic mice in which human estrogen receptors, either normal or superactive, under the control of the keratin 14 gene promoter were present in the basal cells of the lower reproductive track and mammary gland epithelia.
- Found that mice with human estrogen receptors superactive towards target genes with AP-1/CRE elements in the cervix and vagina had hyper-proliferation of the epithelial cells of the lower reproductive track.
- Have preliminary evidence that mice with AP-1/CRE superactive human estrogen receptors in the mammary gland basal epithelia have abnormal lobular-alveolar development with exaggerated response to estrogen.
- Created transgenic mice in which human estrogen receptors , either normal or superactive, under the control of the MMTV promoter were present in the luminal cells of the mammary gland.
- Initiated studies of the mammary development of mice with human estrogen receptors in the luminal cells of the mammary gland.

Reportable Outcomes:

Manuscripts:

Submitted- **An estrogen receptor that is superactive at alternative response elements causes hyper-proliferation** (under revision for Genes&Development)

In preparation- **Proliferative abnormalities in mammary glands of transgenic mice that express a human estrogen receptor superactive at alternative response elements.**

Presentations:

Invited talk. Estrogen pathways to proliferation. Frontiers of estrogen action meeting sponsored by Wyeth-Ayerst, Ireland April 2001.

Invited talk. Estrogen receptors and proliferation. Joint UCSF Breast and Prostate Cancer Special Programs in Research Excellence meeting. March, 2002.

Invited talk. Roles of estrogen receptors in proliferation. Merck and Co. Raway, NJ. April 2002.

US Patent applied for:

Expression of Human Steroid Receptors in Transgenic Animals. University of California Case No. 99-382-1. US Patent Application No. 09/365,614

Development of animal models:

Developed transgenic mice with expression of human estrogen receptor alpha either wild type or AP-1/CRE superactive driven by the keratin 14 promoter or the MMTV promoter with expression in skin, mammary gland basal and luminal epithelial cells, and vaginal-cervical epithelial cells. These mice will be useful to anyone studying the effects of estrogens, including environmental estrogens, on the induction of breast and reproductive track cancers.

Funding applied for:

We have applied for funds to continue and expand the characterization of the above mice from NIH. This is part of a pending competitive continuation of "Gene activation by antiestrogens used in cancer therapy" R01 CA 80210

Richard Price, who has done much of this work has also applied for funds from the California Breast Cancer Research Program to continue his work. It appears that his application will be funded.

Conclusions

We have been successful in developing transgenic mice with expression of wild type and AP-1/CRE superactive human ERαK206A in mammary gland and reproductive track (vaginal-cervical) epithelium. We use d the keratin 14 gene promoter to drive expression in mammary basal epithelial cells and cervical- vaginal epithelium, and the MMTV promoter to drive expression throughout the mammary epithelium. In the K14 transgenics, expression of the superactive receptor in the genital tract was efficient and caused hyperproliferation, cyclin D1 over-expression and organ enlargement. Expression of the superactive receptor in the basal cells of the mammary gland was weak, yet it also caused proliferative abnormalities. These results point to a role for epithelial estrogen receptors and the AP-1 pathway in epithelial proliferation.

We tentatively conclude that human ERα can function to mediate proliferation in the epithelial cells of the reproductive track and the mammary gland, and that the target genes with AP-1/CRE elements are important in this process. If further studies confirm these observations it will suggest that the ERα pathway to AP-1/CRE target genes is a key target for interventions to prevent breast cancer.

"so what section"

Expressing the K206A mutant human ERa, which is selectively superactive towards AP-1/CRE target genes, in the mammary gland appears to lead to hyperproliferation. Since the wild type human ERa does not produce hyper-proliferation, it appears that estrogen action leading to AP-1/CRE regulated target genes may be the pathway leading to proliferative response. If future studies confirm these observations it will have applications in the development of therapies and preventatives for breast cancer, in which estrogen exposure is the major risk factor. Once we know the pathway, we can take steps to block hormone action, and maybe some day develop better antiestrogens to treat or prevent breast cancer.

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Appendices

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